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Age- and Experience-Related Distribution of Parvalbumin-Expressing GABAergic Interneurons within the Hippocampus & Surrounding Cortices

Jennifer A. Corriveau
University of Connecticut - Storrs, jennifer.corriveau@uconn.edu

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Jennifer Ann Corriveau

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Presented by:

Jennifer Ann Corriveau, B.A.

Major Advisor: _____

Dr. James J. Chrobak, Ph.D.

Associate Advisor: _____

Dr. John D. Salamone, Ph.D.

Associate Advisor: _____

Dr. Chi-Ming Chen, Ph.D.

University of Connecticut

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Chapter One: Introduction

Goals of Current Research

The present study sought to elucidate upon the paucity of findings currently available with regard to age-related differences in parvalbumin (PV) expression across brain regions and the septotemporal axis of the hippocampus (HPC) in rats. The lack of available information regarding normative distribution of this calcium binding protein is surprising, given the multitude of studies demonstrating: 1) decreased distribution of PV-expressing GABAergic neurons in post-mortem tissue from schizophrenic patients, and 2) a decrease in PV-expressing GABAergic neurons in forebrain tissue subsequent to acute and chronic NMDA antagonist treatment (e.g., Kittelberger et al., 2012; Wang et al., 2008; Abekawa et al., 2007; Braun et al., 2007; Keilhoff et al., 2004). The latter of which is a widely used model to examine the neuropathological events, as well as the altered behavior/cognitive processes, seen in schizophrenia.

In chapter one, we provide context for the current study, first with a description of the features of schizophrenia and the anatomical changes observed in post-mortem human brain tissue. Secondly, we discuss the importance of observed changes in local circuit GABAergic neurons, and define the anatomical and morphological features of key GABAergic cell populations. Lastly, we discuss the literature regarding treatment- and age-related changes in PV expressing neuron populations.

Schizophrenia

Schizophrenia is a complex psychological disorder affecting approximately 1% of the population worldwide (Perälä et al., 2007). The illness is characterized by positive symptoms including hallucinations, delusions, and paranoia; negative symptoms which include social deficits and flattened affect; and, of particular note, cognitive symptoms which can manifest as marked deficits in attention and memory (Lewis & Lieberman, 2000; Gold, 2004). Currently, positive and, to a certain extent, negative symptoms can be managed through pharmacological treatment with antipsychotic medication such as haloperidol, risperidone, and clozapine, to name a few (Carman et al., 1995; Leucht et al., 2009). However, there are no antipsychotic pharmaceutical interventions capable of mediating the debilitating cognitive deficits characteristic of schizophrenia (Buchanan et al., 2007). Within the past two decades, there has been a concerted effort to understand the underlying etiology of cognitive deficits in schizophrenia. In order to better understand these deficits, and thus foster development of novel cognitive-targeting interventions, it would be helpful to establish a more comprehensive understanding of the neurobiology of forebrain circuits that support the cognitive processes which are altered in schizophrenia.

There are many theories regarding the etiology of schizophrenia, including, but not limited to: genetic predisposition (Bergen & Petryshen, 2012; Kukshal et al., 2012; Owen, 2012; Greenwood et al., 2013), prenatal infection (Brown & Derkits, 2009; Brown & Patterson, 2011), environmental influence (van Os et al, 2010; Bourque et al., 2012), as well as a large body of evidence

supporting combinations of the aforementioned (e.g. Clarke et al., 2009; Holtzman et al, 2012; Pelayo-Terván et al., 2012). However, it is clear from imaging and postmortem studies that distinct anatomical changes – including: volumetric (Bogerts et al, 1985; Honea et al., 2005; Haijma et al, 2012), circuitry (Lewis & Sweet, 2009; Uhlhaas & Singer, 2010), connectivity (Camchong et al., 2011; Schmitt et al., 2011), and neuronal (Harrison, 1999) - contribute to the core cognitive dysfunction in schizophrenia. Importantly, forebrain circuitry, including, but not limited to, prefrontal cortex and temporal lobe structures, are found to have altered regional activity (e.g. magnetic resonance imaging; Shenton et al., 2001; Schobel et al., 2009), differences in white matter connections (e.g. diffusion tensor imaging; Kubicki et al., 2007), and regionally specific volumetric decreases (Sigmundsson et al., 2001) in schizophrenic patients as compared to healthy controls.

Circuitry Implicated in Schizophrenic Cognitive Dysfunction

Cognitive dysfunction is pervasive throughout the lifetime of schizophrenic patients, and severity of cognitive deficits is independent of other psychotic symptoms, and even predictive of prognosis (Tamminga et al., 1998). Much emphasis has been placed on the decreased ability of patients to complete working memory tasks, particularly those involving a high degree of information manipulation (Barch, 2006). Dorsolateral prefrontal cortical (DLPFC) circuitry is implicated in these working memory deficits, and studies of working memory in schizophrenics have even demonstrated an alterations in DLPFC activation in

patients during working memory tasks (Tan et al., 2007). Additionally, hippocampal circuitry, as well as hippocampal-cingulate cortical circuit systems, is thought to be altered at both macro- (e.g. regional interconnections) and micro- (e.g. local inhibition onto pyramids) levels (Benes, 2000). Within these circuits, we are particularly interested in changes seen in GABAergic local circuit networks, as they play a critical role in regulating cognitive and memory processes (Buzsaki & Chrobak, 1995). Furthermore, evidence suggests that there are evolutionary changes within select GABAergic neurons within the forebrain contribute to developmental alterations in cortical and hippocampal macro- and micro-circuits (Hof et al., 1999; Burns, 2004). These alterations can manifest as cognitive dysfunction in a number of psychiatric disorders (e.g., schizophrenia, autism, Alzheimer's; Raghanti et al., 2010).

Alterations in activation seen during neuroimaging studies in DLPFC are likely due to dysfunctional neuronal activity. Specifically, a decrease in GABAergic neurotransmission, and consequently decreased inhibition of excitatory pyramidal neurons, and conversely, even increased inhibition due to compensatory up-regulation of GABA receptors (Gonzalez-Burgos & Lewis, 2008), have been described (Volk & Lewis, 2002; Lisman et al., 2008). Of particular note, the selective decrease of PV-expression within inhibitory GABAergic interneurons, especially within prefrontal cortex and hippocampus, is thought to play a key role in these observed alterations (Lewis et al., 1999; Hashimoto et al., 2003; Lewis et al., 2004). Disrupted signaling within this circuit is likely the driving force behind these deficits, and therefore inhibitory

interneurons are being considered as targets for possible cognitive-enhancing treatments (Lewis et al., 2005).

Afferent and efferent connections between the DLPFC and hippocampus are also thought to be altered, thereby contributing to deficits seen during cognitive processes that require the manipulation and temporal and/or spatial encoding of information (Meyer-Lindenberg et al., 2005). Furthermore, local inhibitory circuitry within the hippocampus has been found to be altered at the synaptic level (e.g. aberrant functionality and synaptic connections) and contributes to widespread cognitive dysfunction (Harrison, 2004). The GABAergic alterations within the prefrontal cortex and hippocampus are further implicated in post-mortem studies of schizophrenic brains, where immuno-reactive labeling studies reveal a selective reduction of the calcium-binding protein parvalbumin, relative to controls (Beasley & Reynolds, 1997; Zhang & Reynolds, 2002). While there is a selective reduction in PV-expressing GABAergic neurons, there is no concurrent decrease in either calretinin or calbindin populations (Zhang & Reynolds, 2002; Lewis et al., 2012). Why forebrain PV-expressing GABAergic neurons would be selectively disrupted is unclear, although evidence presented in the current study suggest developmental changes in PV expression that perhaps make these neurons vulnerable to dysfunction.

GABAergic Inhibitory Interneurons

GABAergic interneurons are integral for optimal functioning within the brain, and play important roles in regulating neurotransmission and coordinating concerted neuronal ensembles (Buzsaki & Chrobak, 1995). These interneurons can be divided into various subtypes, often by the calcium-binding proteins they express (McBain & Fisahn, 2001). Within the brain, there are many different populations of inhibitory interneurons expressing calcium-binding proteins, some of which are used as cellular markers, including: calbindin, calmodulin, calretinin, and parvalbumin (Freund & Buzsaki, 1996; Markram et al., 2004). These calcium-binding proteins allow for the binding and intracellular transportation of calcium ions and can differentially affect the temporal dynamics of cellular function post stimulation (Arif, 2009).

Parvalbumin-Expressing Interneurons

Parvalbumin-positive interneurons are widespread throughout the brain and can be subdivided by their morphological characteristics, as well as their laminar and anatomical locations (Freund & Buzsaki, 1996; Hof et al., 1999). PV protein is primarily expressed in three distinct GABAergic interneuron subtypes: basket cells, chandelier cells (in neocortex; referred to as bistratified cells in HPC), and O-LM cells (in stratum oriens in HPC) (Freund & Buzsaki, 1996; Klausberger, 2009). Chandelier cells are primarily located in cortical layers II-VI, as well as within the hippocampus (though here they are often referred to as axo-axonic or bistratified cells), and have axonal terminations exclusively on the axon

initial segment of excitatory pyramidal neurons (Markram et al, 2004). Though there are relatively few of this cell type compared to other inhibitory interneurons, these are thought to be very powerful with regard to their ability to regulate neural transmission in the brain (Woodruff et al., 2010). Due to their inhibition directly onto the axon initial segment, chandelier cells also play an important role in regulating pyramidal cell excitatory output (Lewis et al., 2004); specifically, the ability for a single chandelier cell to target large populations of pyramidal neurons allows for wide-spread synchronization of excitatory transmission (Cobb et al., 1995).

Basket cells, which are located within the cortex, hippocampus, and cerebellum (Seto-Ohshima et al., 1989), synapse onto pyramidal cell somata and proximal dendrites and are primarily located within the pyramidal and granule cell layers in the hippocampus (Nitsch et al., 1990; Freund & Buzsaki, 1996). Like chandelier cells, a single parvalbumin-containing basket cell is able to make a large number of connections onto local pyramidal neurons, as well as synapsing with other parvalbumin-containing interneurons (Sik et al, 1995), thereby giving these interneurons the ability to strongly modulate postsynaptic excitatory output (Aradi et al., 2002). PV expressing cells are also located outside of the pyramidal and granule cell layers within stratum oriens, where these PV-expressing interneurons are referred to as O-LM cells, as well as in the hilus (Sloviter, 2004).

Within the CA1 region of the hippocampal formation, basket cells and chandelier cells make up approximately 75% of the total interneuron population (Szilagyi et al., 2011). The anatomical distribution of parvalbumin-positive

inhibitory interneurons within each sub-area of the hippocampus (CA1, CA3, dentate gyrus) appears to have consistent laminar profiles across the dorso-ventral axis, as well as no dorso-ventral differences in expression within each hippocampal area, at least in the rat (Kosaka et al, 1987; Nomura et al, 1997a; 1997b).

Treatment and Age-Related Changes in Parvalbumin Expression

Of particular relevance to the selective decrease in PV expressing interneurons within schizophrenic postmortem tissue, have been studies employing acute and/or chronic NMDA-antagonist drug regimens in rodents (e.g. Kittelberger et al., 2012). While such studies have generally reported a decrease in PV as a result of NMDA-antagonist administration (e.g. Romon et al., 2011; Wang et al., 2008; Braun et al., 2007; Keilhoff et al., 2004), there have been some studies indicating no change in PV-immunoreactivity following drug administration (e.g. Benneyworth et al., 2011; Zhang et al., 2008). What is interesting regarding these conflicting findings is that the studies do not control for age as a possible factor influencing PV expression. Rather, many of these studies seem to assume stability in PV expression over the lifespan. A major rationale for the current studies was to examine age-related differences in PV expression so as to better understand the existing literature and inform future studies on the effects of chronic NMDA-antagonist treatments.

Age-related changes in parvalbumin expression have been found in cortical (Ouda et al., 2008), as well as subcortical areas including the

hippocampus (Vela et al., 2003). However, there have been very few studies offering conclusive findings outlining age-dependent changes in hippocampal PV expression in a systematic way. In fact, to our knowledge, only a few studies have been conducted seeking to directly compare PV expression in young and aged rats in an attempt to characterize calcium-binding protein distribution as a function of age(e.g. Shetty & Turner, 1998; Vela et al., 2003; Stanley et al., 2012). Though there have been few studies, Shetty and Turner (1998) describe significant decreases in PV immunoreactivity within HPC CA1, CA3, and hilus between 5- and 24-month old rats, and these findings are in agreement with the limited work conducted across portions of the rat lifespan (Vela et al., 2003; Stanley et al., 2012).

The lack of systematically conducted normative baseline data regarding PV distribution across the lifespan is concerning, especially considering that rodent studies examining PV expression rarely account for the age of the animals used. This is evident in a study conducted by Nomura and colleagues (1997a) reporting no changes in hippocampal PV expression. Though they looked both between hippocampal regions and across the septotemporal axis, their data was exclusively collected from 5 week old rats, and therefore should not be generalized as a stable property of PV distribution across all age groups. Furthermore, the lack of baseline data makes it difficult to make conclusions based on data derived from experiments utilizing drug manipulations, as PV immunoreactivity may be dynamically altered based on a variety of endogenous and exogenous factors including, but certainly not limited to, age and experience.

The current study aimed to provide the first systematic characterization of PV-expression within the hippocampus, as well as within somatosensory and limbic cortices. In order to address the lack of data on normative PV distribution we set out to determine the following things with regard to PV immunoreactivity in young, adult, and aged rats: 1) whether PV expression differs along the septotemporal axis of the hippocampus; 2) whether or not PV exhibits an age-dependent profile of distribution and to determine whether our findings were consistent with the few studies which have also looked at age as a key factor; and 3) whether PV expression varies as a function of hippocampal sub-region (CA1, CA3, and DG), as well as cortical region (somatosensory and limbic cortices). The current study provides key normative data to inform future studies employing experimental manipulations, and allows for a more dynamic view of PV as a neural marker for pathology.

Chapter Two: Manuscript

Abstract

Parvalbumin (PV)-expressing inhibitory interneurons are the most numerous GABAergic interneuron type in the neocortex and hippocampus (HPC). Selective reductions in PV interneurons are observed in cortical and limbic regions in postmortem human schizophrenic brain tissue. Reductions in PV expression are also observed in rodents following acute and/or chronic treatment with NMDA-antagonists (e.g. ketamine). There are inconsistencies in the literature regarding age-related changes in PV expression in the HPC; some stating age-related decreases, no observed changes, and even increases in PV-immunoreactivity. These inconsistencies make it difficult to qualitatively and quantitatively evaluate changes observed in rodent models following NMDA-antagonist treatment. In order to provide a better baseline understanding of putative developmental changes in normal PV expression, the current study quantified 1) age-related changes in HPC and cortical PV expression in rats; 2) whether PV varied along the septotemporal axis of the HPC; and 3) regional changes in HPC PV expression. PV immunohistochemical methods were used to examine PV expression in hippocampal and adjacent neocortical regions in 1 and 6 month old naïve, as well as 24 month old behaviorally trained (radial water maze training), male Sprague-Dawley rats. Our main findings include: 1) specific regional differences (CA1, CA3, DG) across the septotemporal axis that varied by developmental age; 2) age-related decreases in PV expression in CA1 and DG (50-68% and 88-89% decrease from 1 – 6 months, respectively) in naïve rats;

and 3) A prominent increase in PV expression in the 24 month old rats that may be related to age or behavioral experience. These findings demonstrate developmental differences in the regional expression of PV, as well as potential behavioral and experiential increases in PV expression. Importantly, these baseline data provide key information for future work examining age-related differences in PV as a consequence of acute or chronic NMDA-antagonist treatment.

Introduction

A consistent finding in post-mortem schizophrenic brain tissue has been a selective reduction in a subset of inhibitory GABAergic interneurons containing the calcium-binding protein parvalbumin (PV) (Beasley & Reynolds, 1997; Volk et al., 2012; Lewis et al., 2012). PV-containing interneurons provide critical inhibition onto the soma and proximal dendrites of excitatory pyramidal neurons (Zuschratter et al., 1985; Kawaguchi et al, 1987; Katsumaru et al, 1988; Williams et al., 1992), which allows them to play a key role in determining the excitatory output of their postsynaptic targets (Lewis et al., 2004). This role likely contributes to the ability for involved neuronal circuits to accurately relay relevant information and support neural circuit processes key to cognitive function. Because of their importance in neocortical and hippocampal neuronal circuitry, the decrease in PV interneurons seen in schizophrenic brains likely contributes to the cognitive dysfunctions observed in schizophrenia.

Research of the underlying etiology of schizophrenia is difficult, as post-mortem human tissue can be confounded by countless variables (e.g., medication, co-morbid illnesses, drug use, etc.). Therefore, rodent models have been integral in enhancing our understanding of the proposed neural mechanisms underlying the disorder. A multitude of rodent models exist for the study of schizophrenia, ranging from genetic knockout mice (Chen et al., 2006), social isolation (Stevens et al., 1997), post-natal (Wedzony et al., 2008) or adult drug administration (Egerton et al., 2005; Chrobak et al., 2008), and even those employing a “two-hit” model consisting of two separate insults over the lifespan

(e.g., Bayer et al., 1999; Corriveau & Glenn, 2012). Of particular interest are rodent models exhibiting NMDA-antagonist induced selective reductions in PV expression in key brain regions such as prefrontal cortex and hippocampus (Keilhoff et al., 2004; Morris et al., 2005; Abekawa et al., 2007; Zhang et al., 2008). However, these findings often lack consistency, with some researchers indicating no changes in PV expression (Benneyworth et al., 2011), and other reports indicating exactly the opposite findings of one another (see Benneyworth et al., 2006; table 3). Alarmingly, there have been few studies providing consistent information regarding what could be considered 'normal' with regard to PV distribution within rodent brains, and whether the changes we are seeing are truly representative of human schizophrenic pathology.

In order to better understand the normative distribution of PV interneurons throughout the brain in rodents, and to establish a baseline of PV distribution consistent with past findings, we conducted a series of immunohistological studies in rats to visualize PV expression in cortical and hippocampal areas. We also quantified PV expression across the rat lifespan (specifically in post-weaning, young adult, and aged cohorts) within the brain regions of interest, as there are mixed reports indicating a decline in PV expression in some (Lolova & Davidoff, 1992; Miettinen et al., 1993), but not all (e.g., Vela et al., 2003; Potier et al., 2006), age-related PV studies. The current study also looked across the septotemporal axis of the hippocampus across aging, as there have been no studies, to our knowledge, that have systematically examined changes in PV expression in such a way. Here, we provide compelling evidence for an age-

dependent reduction of PV expression across hippocampal areas, as well as age-dependent septotemporal variation in PV expression. Furthermore, we provide evidence suggesting a role for behavioral enrichment in increasing PV expression within the rodent brain. Overall, our findings support a dynamic role of PV expression across the rat lifespan, particularly within the hippocampus and, to a lesser degree, within adjacent limbic cortex and neocortical regions.

Materials and Methods

Animals

Twenty-six male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) spanning three different ages were used: adolescent (approximate age P30; referred to as 1 month old; *n* = 8), young adult (approximate age P120-180, referred to as 6 month old; *n* = 8), and aged (24 months old, *n* = 8). Young adult and aged rats were single-housed, while adolescent rats were housed in groups of four, in clear polycarbonate caging. Normal husbandry procedures were followed for all rats within a temperature and humidity controlled vivarium, with a 12-hour light/dark cycle (lights on at 0800h), with access to food and water *ad libitum*. Additional animals were used to optimize Parvalbumin (PV) antibody concentration prior to immunohistochemical processing of experimental tissue (*n* = 2).

Young (1 month) and adult (6 month) rats were experimentally naïve, while aged (24 month) rats had a history of behavioral testing (continuous radial

water maze experience from approx. P60 – 1.5 years of age; details of training can be found in Chrobak et al., 2008) and pharmacological testing (ketamine and cannabinoid antagonist treatments from 1-1.5 years of age). Additionally, aged rats had restricted food intake strictly during behavioral testing. However, all behavioral and pharmacological manipulations were completed minimally four months prior to sacrifice/brain collection. All housing and experimental procedures were in accordance with, and approved by, the University of Connecticut Institutional Animal Care and Use Committee.

Immunohistochemistry

All rats were anesthetized with Euthasol (pentobarbital sodium solution; 1mL via intraperitoneal injection) and transcardially perfused first with ice-cold .9% physiological saline solution (approximately 200mL for 5 minutes), followed by ice-cold 3.7% paraformaldehyde solution (approximately 200mL for 5 min). Brains were removed and stored in 3.7% paraformaldehyde solution prior to being cryoprotected in a 30% sucrose solution for 48 hours before being sliced into 60µm coronal sections on a cryostat. Sections were taken from approximately -2.5mm through -6.0mm relative to Bregma (Paxinos & Watson, 1997; see Figure 1) that included distinct septotemporal areas of the HPC as well as adjacent cortical areas (retrosplenial and somatosensory), and then stored in phosphate-buffered saline (PBS)-containing well plates.

One series of tissue slices (each sample roughly separated by 240µm) per brain were selected to examine Parvalbumin (PV) immunoreactivity (*n*=24

series). Free-floating sections were initially blocked in 5% normal goat serum (Jackson Immuno), 0.1% triton-X, and PBS solution for 1h, followed by 3 five-minute rinses in PBS. Sections were then transferred into primary antibody solution (1:4000 anti-rabbit parvalbumin antibody (Calbiochem), 0.1% triton-X, and PBS – antibody concentration determined previously using aged rat brains (*n*=2) (see Figure 2)) for a 24h incubation period at room temperature. Sections were then rinsed 3 times at 5 minutes, and transferred into secondary solution (HRP labeled polymer anti-rabbit, DAKO) for a 2h room-temperature incubation period. Following a final set of 3 five-minute rinses in PBS, sections were transferred into a diaminobenzidine (DAB) chromogen solution (DAKO; Carpinteria, CA) for ten minutes to develop the stain. Sections were mounted and dried on glass slides, then cover-slipped for microscopy using DPX. Adjacent sections were processed for Nissl body staining using thionin to assist with identification of brain regions.

Data Analysis

A Nikon Eclipse E600 (Melville, NY) upright microscope equipped with an Insight SPOT digital camera (Diagnostic Instruments, Inc.) was used to examine and photograph sections with the assistance of SPOT software (SPOT Imaging Solutions; Sterling Heights, MI). Sections containing the major hippocampal subdivisions (CA1, CA3, and DG) were selected for analysis along the septotemporal axis within -2.45mm through -3.9mm (septal regions) and within -5mm through -6mm (midseptotemporal and temporal regions) relative to Bregma

(Steullet et al., 2010; Nomura et al., 1997a); see Figure 1 for section selection. Additionally, photomicrographs were taken of retrosplenial and somatosensory (barrel field) cortices, sampled from sections containing septal hippocampus, and were digitally catalogued for later analysis (see Figure 10A). Hippocampal sub-regions and retrosplenial cortex were photographed at 10x magnifications, while neocortical areas were photographed at 4x magnification to ensure inclusion and visualization of all cortical layers.

Digital photographs were analyzed using ImageJ software (NIH), and the number of PV immunoreactive interneurons was quantified using macros written to automate particle counting within each specified region. The sizes of the analyzed regions were as follows: CA1/CA3/ DG, 800µm x 500µm; retrosplenial cortex, 800µm x 800µm; somatosensory, 1600µm x 1000µm. Sections were chosen for analysis based on their anatomical location; representative photomicrographs containing septal CA1, CA3, and DG, were taken from levels corresponding to -2.45mm to -3.9mm relative to Bregma (Figure 3A); midseptotemporal and temporal CA1, CA3, and DG were taken from levels corresponding to -5.0mm to -6.0mm relative to Bregma (Figure 3B) (Paxinos & Watson, 1997). The average PV immunoreactivity, as counted using ImageJ macro parameters (see Figure 4), for each counted region was averaged for each age group to determine whether there were differences across hippocampal regions and/or across the long axis, in addition to possible differences within neocortical regions. Regional and areal differences in the distribution of PV+

interneurons were assessed using one-way Analysis of Variance (ANOVA) followed by post-hoc t-tests (Kirk, 2012).

Results

Summary of Results

Overall, data indicated an age (1-, 6-, and 24-month), regional (cortical field, hippocampus), septotemporal (septal, midseptotemporal, temporal), and hippocampal region (CA1, CA3, DG) variation in the distribution of PV-expressing interneurons. First, we will describe the differences in PV expression across the septotemporal axis within the hippocampal subdivisions when comparing 1- and 6-month old rat brain tissue. In 6 month old rats, there was a significant difference in PV expression from septal to temporal CA1 and DG (50- 80% decrease in expression from septal to temporal regions). Second, we describe the age-dependent differences in PV expression observed within specific hippocampal subdivisions. Generally, there were significant decreases in the number of PV expressing neurons in the CA1 and DG regions of 6-month, compared to 1-month, old rats without concomitant changes within the CA3 sub-region. No differences were observed in the two neocortical fields from the same septal coronal sections (barrel field and retrosplenial cortices). Third, we describe the regional (CA1, CA3, DG) differences in PV expression, where there is, generally, a higher density of PV+ interneurons observed in CA1 sub-regions in contrast to CA3 or DG. Lastly, we report a striking increase in PV expression in all regions of interest (cortical areas and hippocampal regions) in the 24-month

old age group, compared to 6-month old rats. Notably, this effect is confounded by the extensive behavioral training that the 24-month old rats received. Ongoing experiments are being conducted to determine whether these differences are consequent of age and/or experience.

Age-related septotemporal variation in HPC parvalbumin expression

There was no difference in the distribution of PV expressing neurons across the septotemporal axis of CA1, CA3, or the DG in 1 month and 24 month old rats (see Figure 5). In contrast, 6-month old rats exhibit a significant effect of septotemporal location in CA1 and DG. Specifically, septal CA1 in 6 month old rats had significantly greater PV expression than both midseptotemporal and temporal CA1 ($F(2, 14) = 21.4$, $p < .001$; see Figure 5A). Similarly, septal DG in 6-month old rats had significantly greater PV expression than the midseptotemporal region ($F(1, 7) = 6.5$, $p < .05$; see Figure 5B). In contrast, there were no septotemporal variations in region CA3 of the hippocampus ($F(2, 14) = 3.3$, $p = .07$; see Figure 5C). For representative photomicrographs of CA1, CA3, and the DG, see Figures 6, 7, and 8 (respectively); descriptive statistics can be seen in Table 1.

1- to 6-months: Age-related difference in regions CA1 and DG that increases with difference from septal pole

Overall, there was a similar expression of PV in region CA3 in 1 and 6 month old rats across all septotemporal levels, however, there was less PV in

more temporal levels in both CA1 and the DG. Specifically, there was an age-dependent decrease in PV expression within CA1, such that 1-month old rats had significantly higher PV expression than 6-month old rats in midseptotemporal ($t(14) = 3.3$, $p < .01$) and temporal levels ($t(13) = 3.8$, $p < .01$), though there was no difference between these groups in septal CA1 t ($p = .24$); see Figure 9A. There was also an age-dependent decline in PV expression in both septal DG ($t(14) = 7.1$, $p < .001$) and midseptotemporal DG ($t(12) = 2.4$, $p < .05$), see Figure 9B. However, no age-related differences in PV expression were observed in septal ($p = .11$), midseptotemporal ($p = .66$), or temporal ($p = .47$) CA3 (Figure 9C). These data can be visualized in representative photomicrographs in Figures 6, 7, and 8. No significant age-related effects were seen in retrosplenial ($p = .52$; Figure 10B) or barrel field ($p = .28$; Figure 10C) cortices between 1- and 6-month old naïve rats (Representative photomicrographs in Figures 11 and 12). Descriptive statistics can be seen in Table 1.

CA1 regions have increased PV expression compared to other HPC regions across all age groups

Significant regional differences in PV expression were seen in septal and midseptotemporal, but not temporal, hippocampal regions across all age groups (1-, 6-, and 24-months). 1-month old rats have significantly higher PV expression in septal CA1 compared to septal CA3 ($p < .01$) and DG ($p < .001$). Midseptotemporal sections follow the same pattern, with CA1 having significantly more PV expression than CA3 ($p < .001$) and DG ($p < .001$). 6-month old rats had significantly higher PV expression in septal CA1 in comparison to septal CA3

($p < .001$) and DG ($p < .001$), in addition to having significantly higher PV expression in midseptotemporal CA1 compared to both midseptotemporal CA3 ($p < .05$) and DG ($p < .01$). In 24-month old extensively trained rats, septal CA1 had significantly higher PV expression than septal CA3 ($p < .01$) and DG ($p < .001$). Interestingly, in midseptotemporal regions, CA1 and CA3 were not significantly different from one another ($p = .25$), but both had higher PV expression than midseptotemporal DG ($p < .01$, $p < .05$; respectively). These data can be seen in Figure 13, and descriptive statistics can be seen in Table 1.

Increased parvalbumin expression in 24-month old extensively trained rats across all hippocampal and cortical regions examined

A striking increase in PV expression was observed in 24-month old extensively trained rats relative to 1- and 6-month old naïve rats within all hippocampal and cortical regions quantified. There was significantly more PV expression in both retrosplenial and barrel field cortices in 24- compared to 1- ($p < .05$, $p < .001$; respectively) and 6- ($p < .05$, $p < .001$; respectively) month old rats (Figure 10). 24-month old rats had significantly higher PV expression than 6-month old rats in all hippocampal regions observed: Septal CA1 ($p < .05$), CA3 ($p < .05$), and DG ($p < .001$); midseptotemporal CA1 ($p < .01$), CA3 ($p < .01$), and DG ($p < .05$); and temporal CA1 ($p < .001$) and CA3 ($p < .01$). 24-month old rats had significantly higher PV expression than 1-month old rats in midseptotemporal ($p < .05$) and temporal ($p = .01$) CA3, but these two age-groups had statistically equivalent PV expression in all other hippocampal regions observed (p's > .1). These data can be seen more clearly in Figure 14, and Table 1.

Discussion

Understanding the normative distribution of a target cellular population - in this case PV expressing GABAergic interneurons - is integral to the interpretation and understanding of data derived from experimental manipulations (e.g., pharmacological, trauma, etc.) in rodent models of psychiatric disorders. Currently, there is a significant lack of normative baseline knowledge of PV expression across the hippocampal septotemporal axis as a function of aging. The current data decreases the paucity of data in this regard and should contribute to future evaluations of PV loss consequent to experimental manipulations. Importantly, we provide compelling evidence of an age-dependent decline in PV expression that varies with respect to hippocampal region (CA1, CA3, and the DG), as well as across the septotemporal axis. Furthermore, we present evidence that PV expression may be more dynamic than previously thought and that extensive behavioral training may actually increase PV expression within the rat brain.

Septotemporal differences in hippocampal PV expression are age-dependent

It is well-known that the hippocampus is integral for cognitive processing and memory formation within the brain (Milner et al., 1998). The hippocampus can be sub-divided along its areal or longitudinal axis into: septal, midseptotemporal, and temporal levels (Amaral & Witter, 1989). These septotemporal regions are also associated with different cognitive, emotional,

and behavioral information processing. Generally speaking, septal regions of the hippocampus receive spatial information in addition to sensory information from the neocortex, while temporal hippocampus favors emotionally salient information processing, due to its increased amygdala input (Bannerman et al., 2004.) Because of these distinct differences in information processing within this structure, it follows that there would also be anatomical markers that vary parallel to functional variation across the septotemporal axis (Gusev et al., 2005; Jinno, 2011).

In the current study, we found clear septotemporal variations in PV immunoreactivity within the hippocampal formation of 6-month old behaviorally naïve rats. Specifically, there was a significant difference in PV expression from septal to midseptotemporal CA1 and DG, with septal areas exhibiting significantly more PV expression than midseptotemporal areas. Conversely, there were no differences in expression between midseptotemporal and temporal CA1 locations. However, this pattern was not observed in the CA3 region of 6-month old rats, nor were there any septotemporal differences in any region for 1- month old naïve and 24-month old behaviorally trained groups. The lack of septotemporal difference within the CA3 region in 6-month old rats suggests the possibility that PV expressing neurons in this region may be more resilient to aging, while CA1 and DG regions may have increased susceptibility to the adverse effects of aging. However, these findings may also indicate a dynamic interaction between aging and septotemporal location within the hippocampus as

well. To our knowledge, this is the first systematic characterization of septotemporal variation in PV expression across distinct age groups.

Previous findings from Nomura and colleagues (1997a) indicate no significant septotemporal variation in PV expression; however, their work was conducted in 5 week old rats (approx.1 month of age) and therefore is in agreement with the lack of septotemporal effect seen in the 1-month old naïve rats in the current study. Conversely, in mouse brains processed to visualize parvalbumin-positive interneurons, research has shown significant dorsal-ventral differences in parvalbumin expression, particularly within stratum pyramidale (Jinno & Kosaka, 2006). More specifically, Jinno and Kosaka (2006) provide evidence of elevated levels of PV expression within CA1 compared to CA3 and DG, which is in agreement with evidence presented in the current study for 6 month old rats.

It is difficult to interpret the lack of septotemporal variation in PV expression within the hippocampus of 24-month old behaviorally trained rats. This is primarily due to the fact that this was the only group that received extensive behavioral training and it is possible that the training influenced the amount of PV expression throughout the brain, including those areas examined presently. Conversely, it is also possible that there is an age-dependent decrease, then subsequent increase, in PV expression. Either way, these results shed light on the dynamic properties of PV protein expression across the hippocampus.

Age-dependent decline in PV expression within the rat hippocampus

Our data provide support for a growing body of evidence describing an age-dependent decline in PV expression within the hippocampus. Work by Nomura and colleagues (1997a) indicate a high degree of PV expression within young (5 week old) rats that is in line with our findings in 1 month old rats of high levels of PV expression coinciding with a lack of septotemporal gradient. However, we observed a significant decline in PV expression as a result of age in CA1 and DG from 1-month to 6-month experimentally naïve rats, suggesting that expression may be developmentally dynamic. These findings are in agreement with previous studies reporting age-related decreases in PV expression within the hippocampus (Kuruba et al., 2011; Lolova & Davidoff, 1992). Importantly, our data argues against previous research indicating an absence of age-related decline (e.g. Kishimoto et al., 1998) or the presence of age-related increases (Choi et al., 2010) in hippocampal and cortical PV expression. Furthermore, the lack of age-dependent effects of PV immunoreactivity in CA3 suggest the possibility that age-related decline in PV expression may be regionally dependent within the rat.

Data from 24-month old rats was not included in the aging analysis because of the anomalous findings which may be a result of the extensive behavioral training these rats received over their lifetime. Generally, 24-month extensively trained rats exhibited equal (if not more) PV expression in comparison to 1-month old rats across all hippocampal sub-regions (Figure 14),

as well as cortical regions (Figure 10). In addition, 24-month old rats had significantly higher PV expression in all hippocampal sub-regions and cortical regions (with the exception of no significant difference in septal CA3, though a clear trend was present; Figure 14). While we provide evidence against an age-related loss of PV immunoreactivity in 24 month old rats, we cannot claim that this is an expected and normative finding in aged rat hippocampus. In fact, in light of the age-related decline in PV expression from 1- to 6-month old naïve rats, we suggest that these findings are an artifact of the extensive behavioral training this group received. Furthermore, we hypothesize that, if these animals had not received a lifetime of behavioral training, their PV expression profile would be that seen currently in the 6-month old naïve rats, if not further decreased, providing strong evidence for PV decrease across the lifespan (see Figure 15 for a proposed timeline of PV expression).

Possible role of extensive training/enrichment in increasing overall PV expression

The prominently high levels of PV expression within all regions of interest in 24-month old extensively trained rats in the present study were surprising and unexpected. While we initially set out to characterize possible age- and regionally-dependent differences in PV expression, we observed consistently elevated expression both within the hippocampal regions (Figure 14), as well as the cortical regions (Figure 10) examined. It is unlikely that these findings are a result of an age-dependent shift in PV immunoreactivity, but rather suggest a

dynamic interaction between the expression of this particular calcium-binding protein within the brain and the behavioral experiences of the animal.

Experience-related changes in the expression of neuronal and cellular markers (e.g., BrdU, NeuN, BDNF, apoptotic markers, etc.) are a well-established phenomenon in the literature (van Praag et al., 2000). A multitude of experiences have the capability of altering brain structure and function, including (but certainly not limited to): maze training (Van der Borght et al., 2005), social interaction (Woolley & Doupe, 2008); physical stimulation (e.g. stimulation of whiskers; Knott et al., 2002); sexual experience (Leuner et al., 2010); stress (Zoladz et al., 2012); and, a topic of extensive research, environmental enrichment (Harati et al., 2011). There are few studies which have examined the effects of enrichment specifically with regard to PV, but those that have been reported indicate enrichment and/or deprivation-related alterations within sensory cortices (Jiao et al., 2006), striatum (De Bartolo et al., 2011), and hippocampus (Iuvone et al., 1996). The findings by Iuvone and colleagues (1996) provide evidence of experience-related increases in PV expression, specifically within the hippocampal regions CA1, CA3, and DG, which is consistent with our findings in 24-month old rats, and suggests that this finding is likely an artifact of enrichment, and not an age-related effect.

General Discussion

Overall, we present evidence in support of age-related decreases in PV-expressing GABAergic interneurons within CA1 and DG regions of the

hippocampus from 1- to 6-month old naïve rats, as well as regionally-specific expression in CA1 compared to CA3 and DG across three distinct age groups. We also provide compelling evidence for a dynamic interaction between PV protein expression within the hippocampus and neocortical regions and life-long enrichment in the form of extensive behavioral training. The present findings provide a framework to inform future studies. Specifically, the regional and age-related differences seen here as a result of age can allow for future experiments seeking to understand the effects of pharmaceutical agents (e.g. NMDA-antagonists), such that they can ensure proper controls, and therefore provide more compelling evidence for differences in neuronal markers. Additionally, the results seen in extensively trained animals open up a new avenue of research, and could possibly inform preventative studies of psychopathologies. Though preliminary in nature, these data provide straightforward evidence for age- and experientially-dependent influences on the expression of PV in rat hippocampus and nearby sensory cortices.

Chapter Three: Discussion

The current study provides support for past research indicating an age-dependent decrease in PV expression within the hippocampus of rats (e.g. Vela et al., 2003). Furthermore, it provides new and compelling evidence for an age-dependent decline in PV expression across the septotemporal axis, which, to our knowledge, has not been previously reported. We found evidence indicating elevated PV expression within the hippocampal CA1 sub-region, compared to CA3 and DG regions, across all age-groups, and a general lack of differences within the CA3 sub-region across age and septotemporal location. Of particular interest, we describe possible experientially-dependent effects on PV expression, indicating that PV has dynamic expression, and can likely be differentially affected by a variety of environmental and biological phenomena. The current study is the first of its kind in systematically characterizing PV expression not only across the septotemporal axis of the hippocampus, but also across diverse age groups in rats, and provides critical baseline data describing PV expression within key brain regions implicated in psychopathology.

Contributions of the current work to the literature: Age-related changes in PV expression

Previous work detailing the distribution of PV expression within the rodent hippocampus and other cortical areas has been important to our understanding of their role in interneuronal circuits. For instance, Nomura and colleagues

(1997a) characterized PV expression across the septotemporal axis of the hippocampus, and indicated no differences in PV expression based on septotemporal level. These findings perpetuate the notion that PV expression is a static marker in GABAergic interneurons within the normal, untreated brain. However, this study lacks generalizability across age groups, as their analyses were only performed in young rats (5 weeks of age). This is problematic for a variety of reasons, but particularly so because many experimental studies (e.g. those using NMDA-antagonist treatments) do not control for age, and baseline data for PV expression across age-groups is needed for comparison and accurate interpretation of results.

An overwhelming degree of inconsistency is seen in the reports of PV immunoreactivity following acute and/or chronic administration of NMDA-antagonist drugs (e.g. ketamine; MK-801). We believe that many of these inconsistencies are likely a result of a failure to control for age in the rodent models utilized, and that their questions may be better answered if they were aware of the dynamic changes seen in PV expression as a consequence of age. For example, studies that have seen no significant changes in PV expression, especially in the hippocampus, following NMDA-antagonist administration regimens have used rodents aged PD7 (Wang et al., 2008), 1 month of age (Zhang et al., 2008), PD 20 & 40 (Stuellet et al., 2010); and 3 months of age (Benneyworth et al., 2011). Note, that all of these studies, which have found no significant impact of NMDA-antagonism on PV expression, have not only used a wide variety of ages across studies, but all of these animals have generally been

3 months of age or less. According to our findings, 1 month old rats appear to have very high expression of PV across all regions studied, and this expression is likely robust and resistant to change in young animals.

Conversely, studies conducted administering NMDA-antagonist drugs in rats 4 months of age and older, observed significant decreases in PV expression within the hippocampal formation (Stuellet et al., 2010; Keilhoff et al., 2004), as well as within the prefrontal cortex (Romon et al., 2011). This disparity is compelling, as it suggests that there may be an age-dependent response to NMDA-antagonist drugs, making adult rodents more susceptible to PV loss as a result of administration of these substances. This could perhaps indicate a decreased stability in the expression of PV in adulthood or, more simply, that PV is a dynamic protein that has the ability to constantly up- or down-regulate its expression with the age and needs of the animal. To make this argument more compelling, studies should be conducted in aged rats (1+ years) in these NMDA-antagonist models to determine whether a similar pattern to adult PV loss is seen, or if perhaps PV decreases are limited to a window of susceptibility, much like what is seen for schizophrenic onset in humans (e.g. Gogtay et al., 2011; Castle et al., 1998). Overall, the current work sheds light on the need for considering age in rodent models of psychiatric disorders to account for the dynamic expression of neuronal markers (e.g. PV) throughout the lifespan.

Contributions of the current work to the literature: Experience-related changes in PV expression

Here we describe a new and intriguing phenomenon of experience-induced increases in PV expression in aged (24-month old) rats. To our knowledge, this is the first report of increased PV in aged rats as a result of extensive life-long behavioral training. While previous studies have observed increases in PV as a result of experience, these results are limited, and generally encompass young animals. For example, reports of increased PV expression within the brain have been attributed to enrichment-related events such as: neonatal auditory stimulation (Chaudhury et al., 2008); increased neural activity in visual cortex (Patz et al., 2004); univibrissa rearing (Nowicka et al., 2009); and adolescent exercise (Gomes da Silva et al., 2010), among others. Furthermore, sensory deprivation in young rodents has been shown to down-regulate PV expression (e.g. Jiao et al. 2006).

Interestingly, a study by Siucinska and Kossut (2006) reports no difference in PV expression in the barrel cortex as a result of short-term sensory learning in adult mice. This is contradictory to our findings of experience-related increases in PV expression within the barrel cortex in 24-month old rats. However, Siucinska and Kossut looked at the effects of short-term sensory learning, while our effect is driven by extensive, life-long behavioral training and experience. Though younger animals seem to have a greater benefit of experience on PV expression, it may be the case that adult and aged animals need more extensive experience in order for it to manifest with increased PV expression. Our data provide

descriptive and quantitative evidence for an up-regulation of PV expression following extensive behavioral training, and can be taken as evidence for the dynamic nature of PV expression across the lifespan, and when faced with different short- or long-term experiences.

Future Directions

While preliminary in nature, the current study has provided us with novel and informative data to move forward with on-going studies in our lab to understand anatomical consequences of chronic NMDA antagonist treatment. Furthermore, the baseline data we have collected through this study provide a starting point for more in-depth future studies to more thoroughly characterize the dynamic nature of PV expression across the lifespan. Of particular note, the data indicating a significant increase in PV expression in extensively trained aged rats has provided rationale for a new avenue of research describing enrichment-induced changes in calcium-binding protein expression.

Expanding upon observed age-dependent changes in PV expression

While the current study provides a starting point for more thorough research into age-related differences in PV expression throughout the rodent brain, there is still much to be done. To our knowledge, there are not studies which have systematically explored PV expression across multiple brain regions as a function of age, and particularly those that look at rats over 1.5 years of age

in comparison to postnatal and adult rats. Such characterization would not only be beneficial to the overall understanding of PV distribution within the rodent brain, but would provide reliable baseline data from which to base future studies aiming to observe PV expression as a meaningful marker of pathology.

In an attempt to further understand the effect of age on PV expression, it would be useful to conduct a series of studies looking at multiple ages of rats including: postnatal pups, young (e.g. 1-3months), young adults (e.g. 6 months), adult (e.g. 1 year) and aged (e.g. 2 years) in order to describe the dynamics of PV expression as a function of age. Furthermore, within each age group multiple brain regions should systematically examined including: hippocampal sub-regions (CA1, CA3, DG, subiculum), somatosensory cortices, limbic cortices, as well as other regions known to have high expression of PV (e.g. cerebellum; De Bartolo et al., 2011). Such an anatomical characterization of PV distribution would add depth to previous studies which have attempted to provide comprehensive understanding of PV distribution, but did not account for age and had a more narrow anatomical focus (e.g. Nomura et al., 1997a; 1997b).

Expanding upon observed enrichment-dependent changes in PV expression

We are especially interested in following up on the enrichment-induced changes observed in the 24-month old rats in the present study. As this is the first report of extensive training resulting in a substantial increase in PV expression (back to levels seen in 1-month old naïve rats and sometimes even

significantly higher), follow-up work is required to validate these results in a more controlled manner. Specifically, we propose to more closely examine the role of enrichment across the rat lifespan to determine 1) whether PV expression is, as is suggest in the present body of work, age-dependent in naïve rats; 2) whether septotemporal variation in PV is age-dependent, as our data imply; 3) whether behavioral experience (in the form of extensive radial water maze training) significantly increases PV expression throughout hippocampus and other areas of the brain (e.g. somatosensory and limbic cortices); and 4) whether an interaction exists between age and experience to differentially modify PV expression in the rat brain. We hypothesize that in naïve rats a significant age-dependent decrease in PV expression would be evident, and that rats receiving extensive training would have no age-dependent decrease in PV expression compared to their naïve counterparts; findings which would be consistent with the data presented in the current study.

Due to the wide use of decreased PV expression as a marker in animal models of schizophrenia (e.g. Kittelberger et al., 2012; Benneyworth et all, 2011), our lab is also interested in the effects of NMDA-antagonism (ketamine) on PV expression and memory as a function of age, as well as experience. Within the radial water maze paradigm (described in Chrobak et al., 2008), we suspect that ketamine-induced memory deficits in the radial water maze may be mirrored by a concomitant decrease in PV expression. Furthermore, since these animals will have had extensive training, and therefore increased PV expression, we believe that any ketamine-induced changes in expression would be clearly evident. In

fact, decreases in PV expression in ketamine-treated behaviorally trained rats are hypothesized to have similar, if not the same amount, of PV expression as their aged experimentally naïve counterparts. Such findings would provide compelling evidence of PV as a dynamic and fluctuating biological marker that has the ability to be influenced by biological, pharmacological, and environmental factors. Furthermore, these proposed findings would also provide validity to previous studies which have observed NMDA-antagonist induced decreases in PV expression (e.g. Kittelberger et al., 2012) and perhaps even offer explanatory evidence for why other studies failed to see a decrease as a result of NMDA-antagonist administration due to their sole use of young rats (e.g. Benneyworth et al., 2011).

General Conclusions

Overall, the current body of work has provided a solid foundation for future work describing age-related and septotemporal changes in PV expression in rodents. Additionally, it has opened up a new avenue of research into the dynamic properties of the calcium-binding protein, PV, and its modulation by behavioral training and enrichment. Though preliminary, our findings provide promising evidence for age- and experientially-dependent changes in PV expression with key brain regions associated with cognitive function. Importantly, the current findings of our study, and those we have proposed, will allow for better understanding, and interpretation, of future studies, especially with regard to modeling schizophrenic cognitive dysfunction.

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Figures

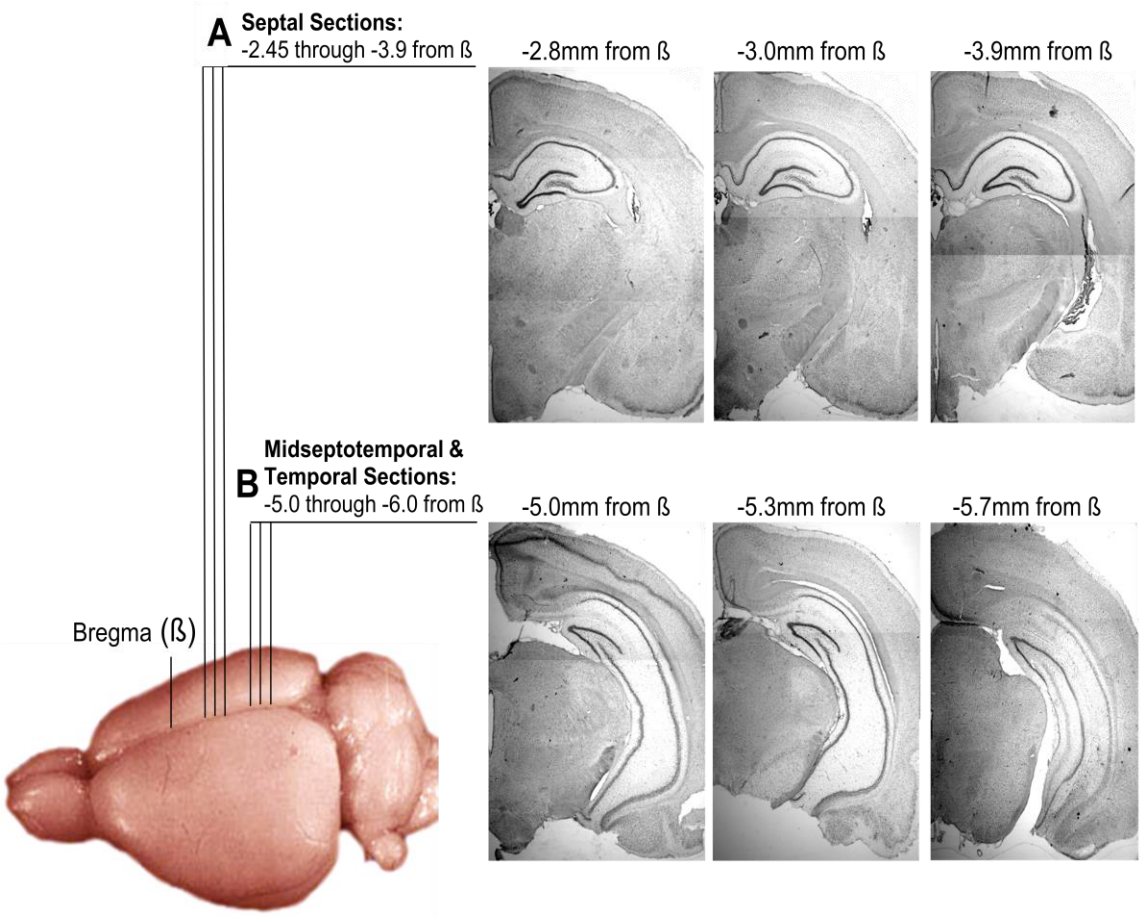


Figure 1: *Location of representative sections relative to Bregma.*

PV-stained sections were pseudo-randomly selected for analysis of PV expression across the long axis of the hippocampus, somatosensory cortex, and retrosplenial cortex. Representative Nissl sections were used to help delineate areas and can be seen in this figure. Septal CA1, CA3, and DG samples, as well as retrosplenial and somatosensory (barrel field) cortex samples, were taken from sections within -2.45mm through -3.9mm relative to Bregma (A). Midseptotemporal CA1, CA3, and DG, in addition to temporal CA1 and CA3, samples were taken from sections within -5.0mm through -6.0mm relative to Bregma (B).Sampled sections were roughly 240µm apart from one another.

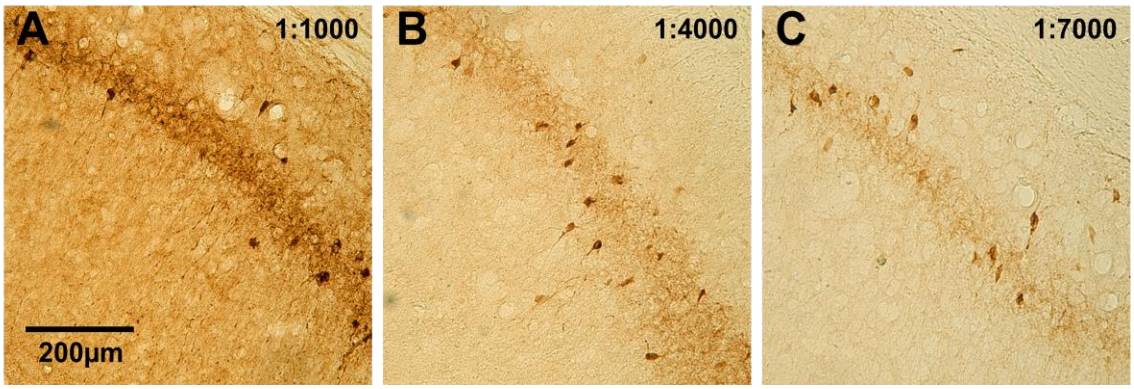


Figure 2:*Optimizing PV Antibody Concentration for Visualization of PV-containing Interneurons.*

In order to determine the optimal anti-parvalbumin antibody concentration, a subset of rats (*n* = 2) were used to compare three different concentrations: 1:1000 (A), 1:4000 (B), and 1:7000 (C). Based on qualitative observations, it was determined that an antibody concentration of 1:4000 was optimal for the purpose of visualizing PV expression for the current study. As evident in A, 1:1000 produced a high amount of background staining making it difficult to distinguish PV-expressing cell bodies. In contrast in C, a concentration of 1:7000 resulted in very light staining, making it difficult to distinguish whether some of the possible expression was, indeed, PV-expressing cells or simply staining artifacts.

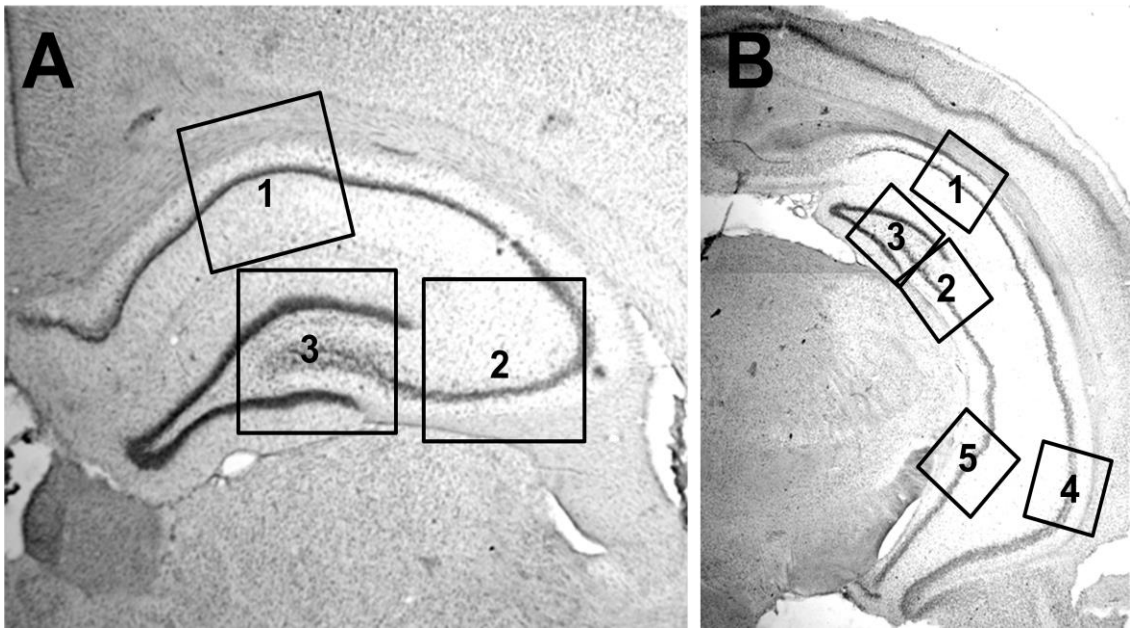


Figure 3: *Anatomical locations of photomicrographs taken for analysis of PV expression in hippocampal regions of interest.*

Photomicrographs of septal hippocampal sub-regions were taken between areas were taken between -2.45mm through 3.9mm relative to Bregma **(A)**, while midseptotemporal and temporal sections were taken between -5mm through -5mm relative to Bregma **(B)**. Septal hippocampal photomicrographs of CA1 **(A.1)**, CA3 **(A.2)**, and DG **(A.3)** were taken at 20x magnification. Midseptotemporal CA1 **(B.1)**, CA3 **(B.2)**, and DG **(B.3)**, as well as temporal CA1 **(B.4)** and CA3 **(B.5)** were also taken at 20x magnification. Photomicrographs of all regions were taken within the boxed regions of interest as outlined above.

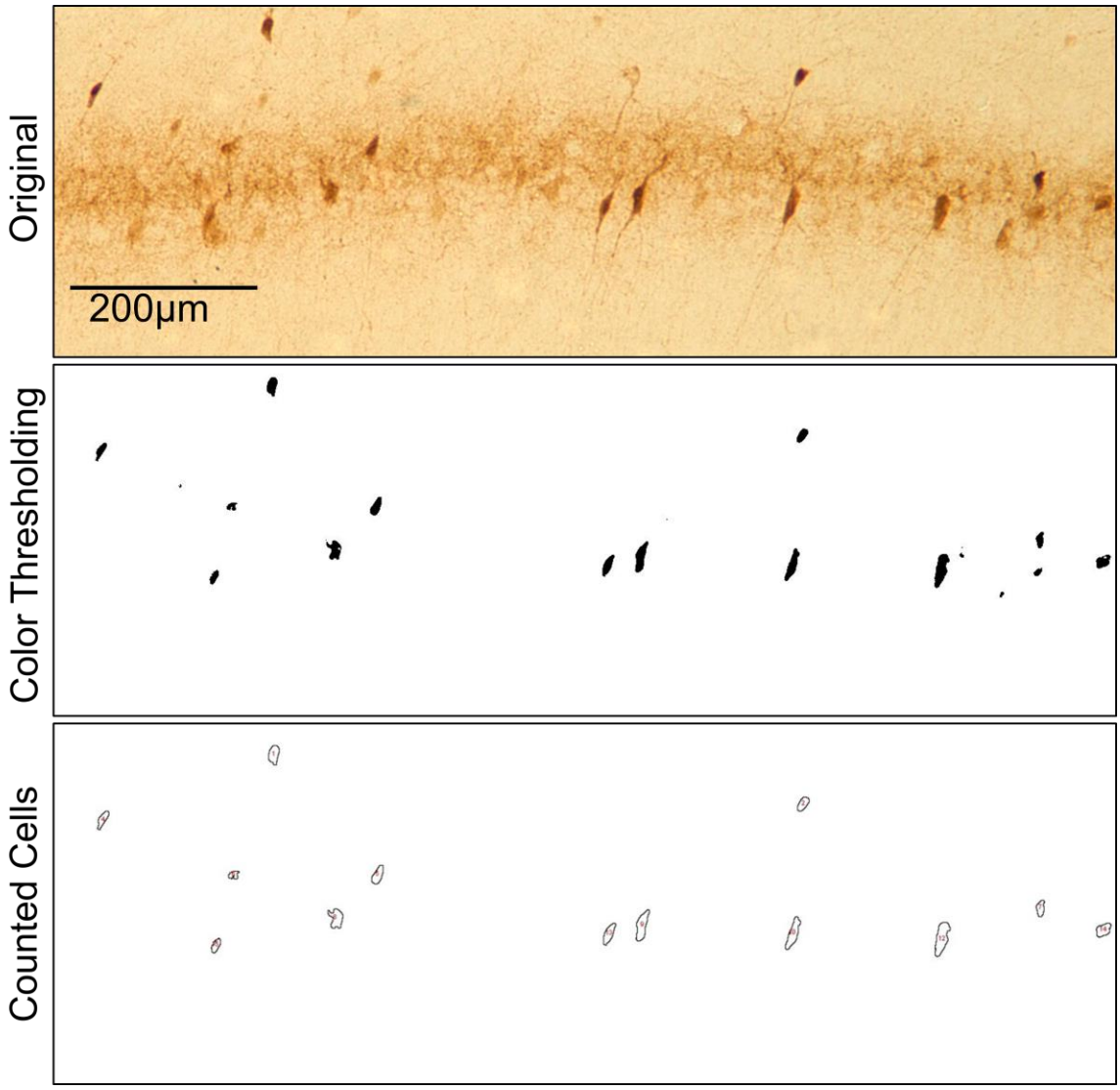


Figure 4: *Example of PV-expressing interneuron quantification using ImageJ color-based thresholding.*

Cell counting was automized using ImageJ and region-specific macros in order to ensure unbiased, consistent quantification within each area of interest. An example of threshold-based quantification, in this case from hippocampal CA1, can be seen above. The first panel shows an original photomicrograph of CA1 with PV-expressing interneurons clearly evident, particularly within the pyramidal cell layer. The second panel shows the same photomicrograph, thresholded based on color density to isolate the most darkly stained cells. A macro was then run to count the number of PV-expressing cells based on previously set parameters and exclude any thresholded particles (e.g. non-cell artifacts) that do not meet criteria for inclusion (e.g. size, sphericity), and provided an output identifying the counted cells (as can be seen in the third panel).

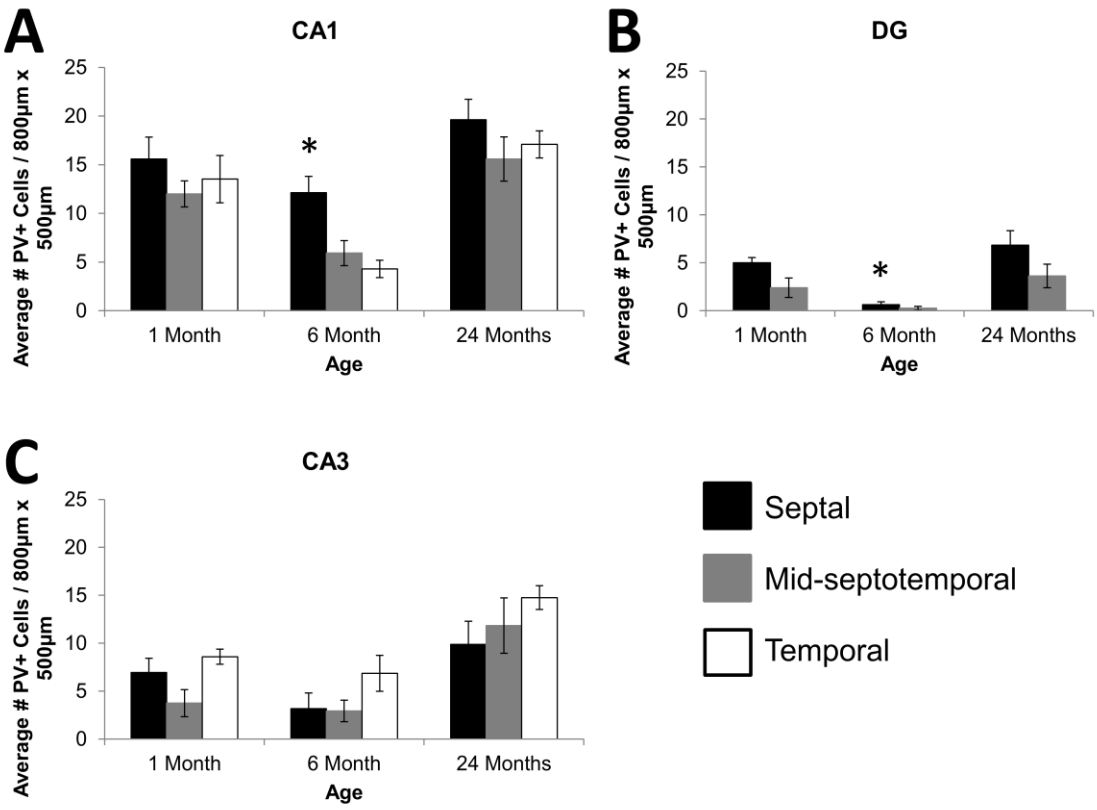


Figure 5: *Septotemporal differences in PV expression as a function of hippocampal sub-region and age.*

6-month old rats had significant septotemporal variation in PV expression within hippocampal CA1 **(A)** and DG **(B)**, but not in CA3 **(C)**. The most striking difference in 6-month old rats were the decreases from septal to midseptotemporal, however, there was no difference between midseptotemporal and temporal CA1 in 6-month old rats **(A)**.There was no effect of septotemporal location in 1- and 24-month old rats in any hippocampal sub-region **(A-C)**.
* $p < .05$

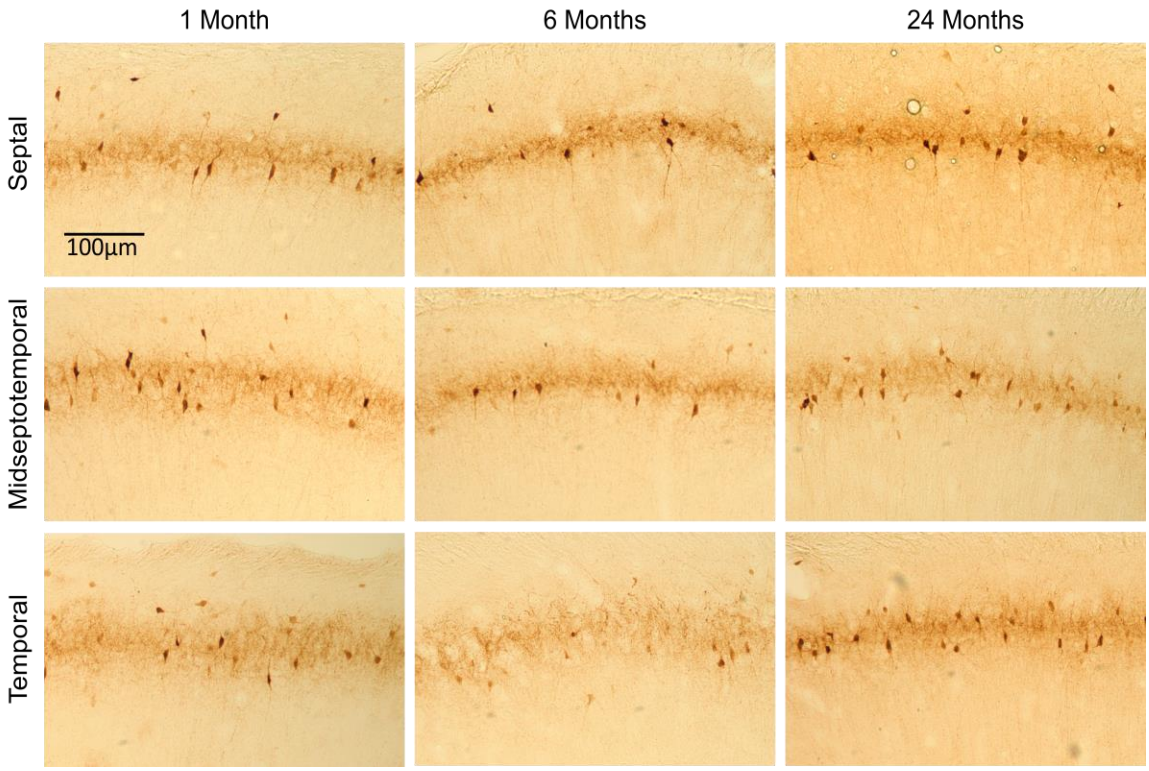


Figure 6: *Distribution of HPC CA1 PV+ interneurons as a function of age and septotemporal location.*

Representative photomicrographs of hippocampal CA1 PV expression across age and septotemporal location. A clear age-related decline in PV-expression from 1- to 6-month old naïve rats in midseptotemporal and temporal CA1 can be seen even through qualitative comparison. 24-month old extensively trained rats have comparatively higher PV expression across all septotemporal levels of CA1.

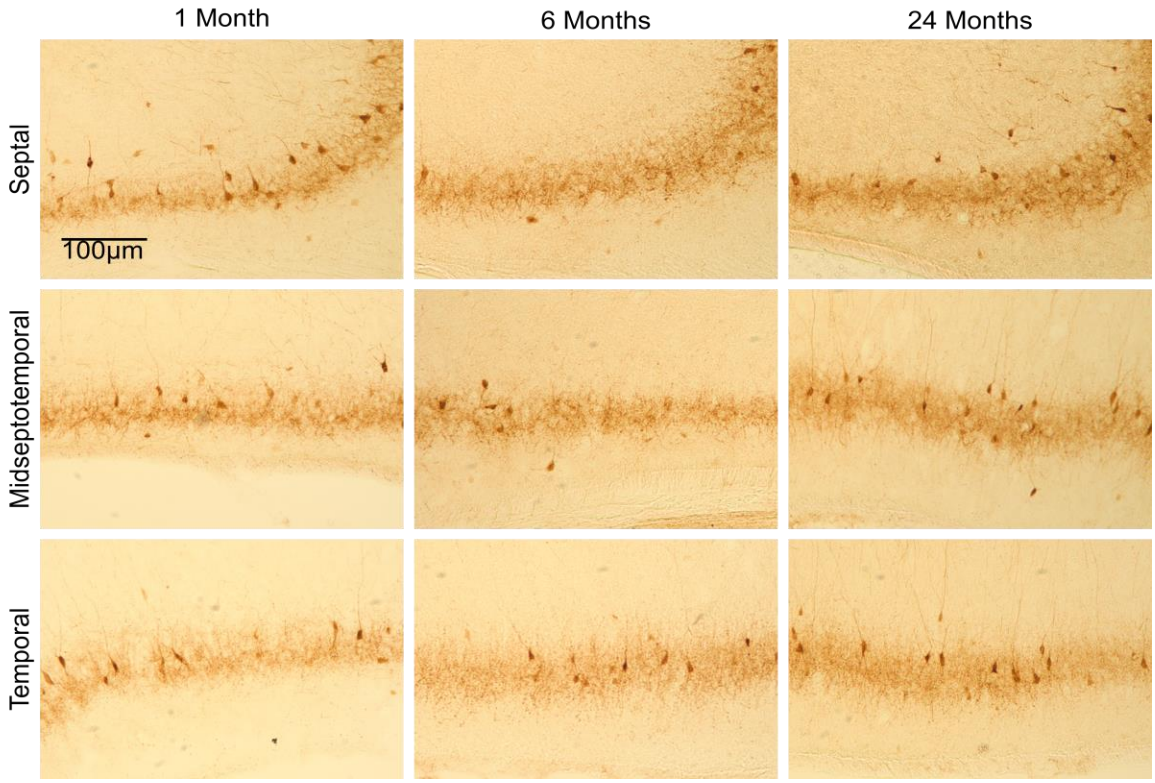


Figure 7: *Distribution of HPC CA3 PV+ interneurons as a function of age and septotemporal location.*

Representative photomicrographs of hippocampal CA3 PV expression across age and septotemporal locations. There were no significant age-related declines in PV expression within any septotemporal level of CA3.

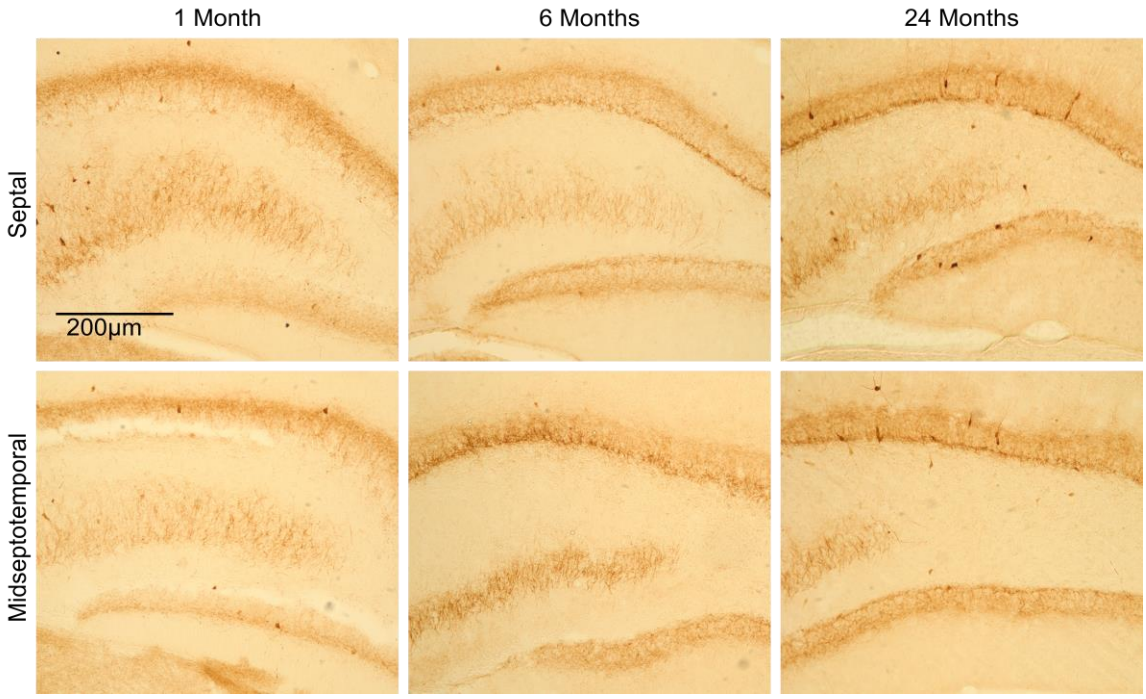


Figure 8: *Distribution of HPC DG PV+ interneurons as a function of age and septotemporal location.*

Representative photomicrographs of hippocampal DG PV expression across age and septotemporal location. From simple observation, it is clear that there are far more PV+ interneurons in extensively trained 24-month old DG. Furthermore, it is evident that there is a significant age-related decline in PV expression from 1- to 6-month old naïve rats.

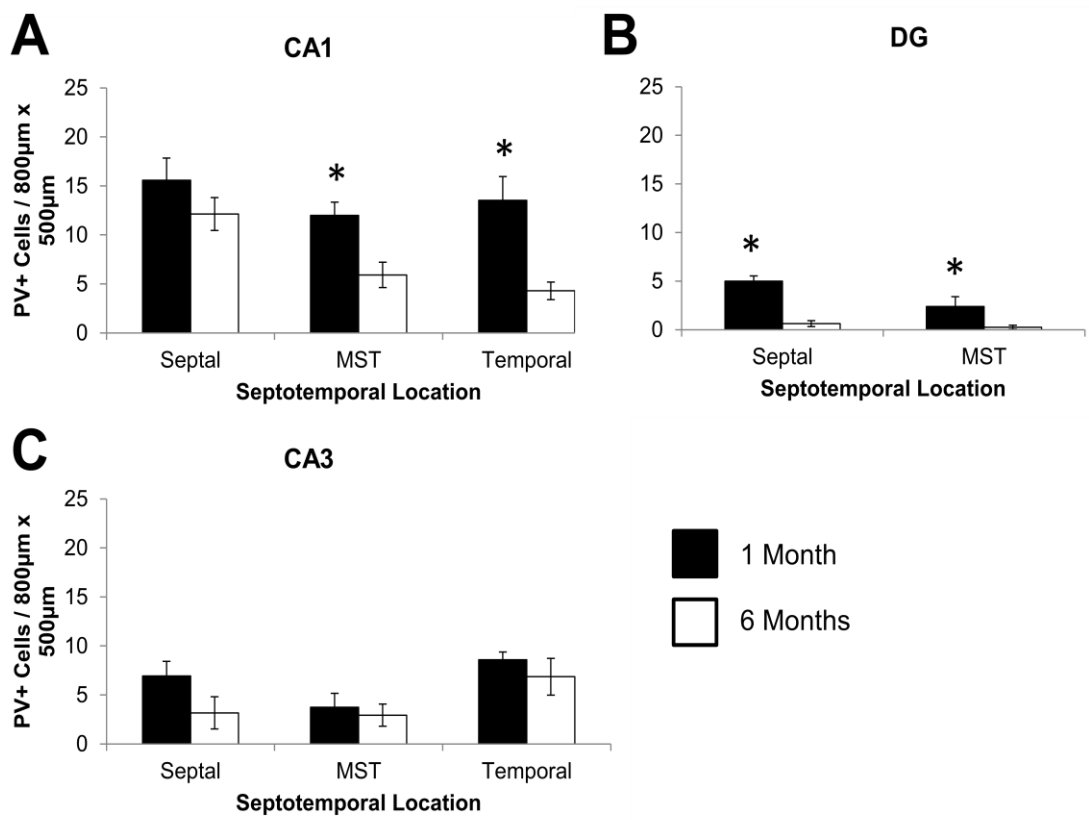


Figure 9: *Age-dependent decrease in parvalbumin expression within the hippocampal sub-regions CA1 and DG, but not CA3.*

Within CA1 **(A)** there is a significant age-related decrease in PV expression from 1- to 6-month old rats in midseptotemporal and temporal locations. This age-related decline in PV expression is also seen in septal and midseptotemporal DG **(B)**. There is no noncommittal decrease in PV expression in any septotemporal region in hippocampal CA3 **(C)**. These data suggest that PV expression decreases within CA1 and DG as age increases. *$p < .05$

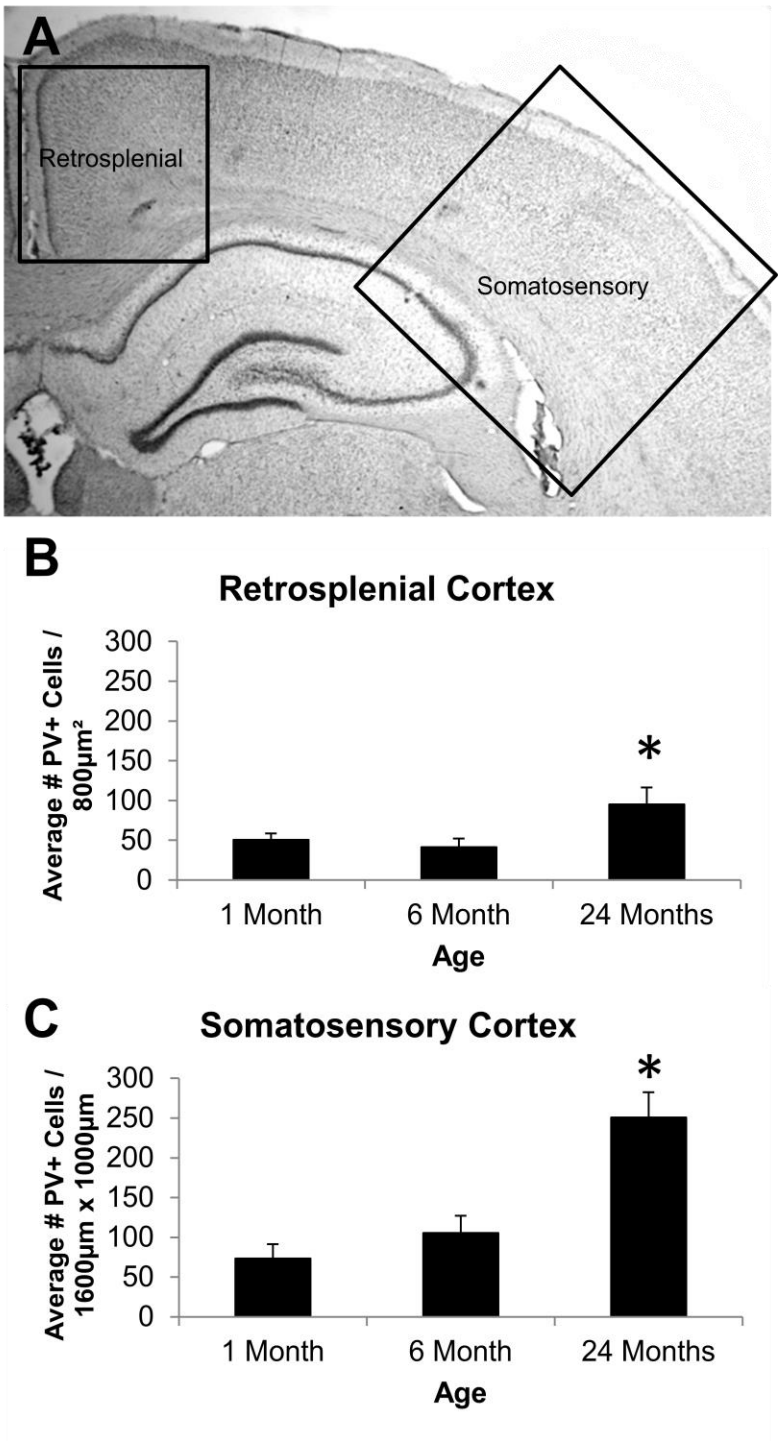


Figure 10: *24-month-old rats have significantly higher PV+ cells within retrosplenial and somatosensory cortices than 1- and 6-month-old rats.*

Photomicrographs of the cortical regions of interest were taken from -2.45mm through -3.9mm relative to Bregma, with retrosplenial cortical photos taken at 20x magnification **(A.1)**, and somatosensory (barrel field) cortical photos taken at 10x

magnification to ensure visualization of all cortical lamina **(A.2)**. 24-month old extensively trained rats had significantly higher PV+ cells within the retrosplenial cortex **(B)** and somatosensory cortex **(C)** than both 1- and 6-month old rats. There was no statistically significant difference between PV expression within these cortices between 1- and 6-month old experimentally naïve rats. These findings suggest that extensive training increases PV+ expression within these cortical regions. *$p < .01$

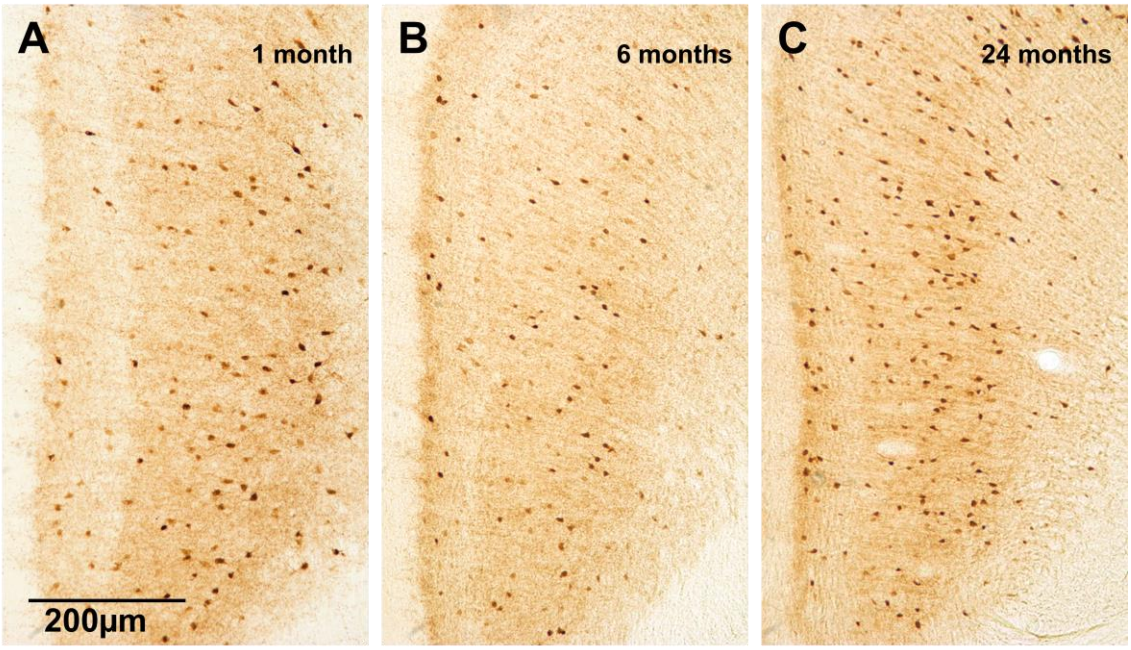


Figure 11: *PV+ expression within the retrosplenial cortex across age groups.*

As shown statistically in Figure 10B, it is clear through visual observation that 24-month old extensively trained rats **(C)** have significantly more PV expression within the retrosplenial cortex than 1- **(A)** and 6- **(B)** month old naïve rats. Though there was no statistically different expression between 1 and 6 month old rats, the PV+ interneurons in 1 month old rats **(A)** appear visually larger than both 6 and 24 month old rats. It is unknown why, though this observation could be due to larger cell size in young rats, or perhaps an increase in PV expression.

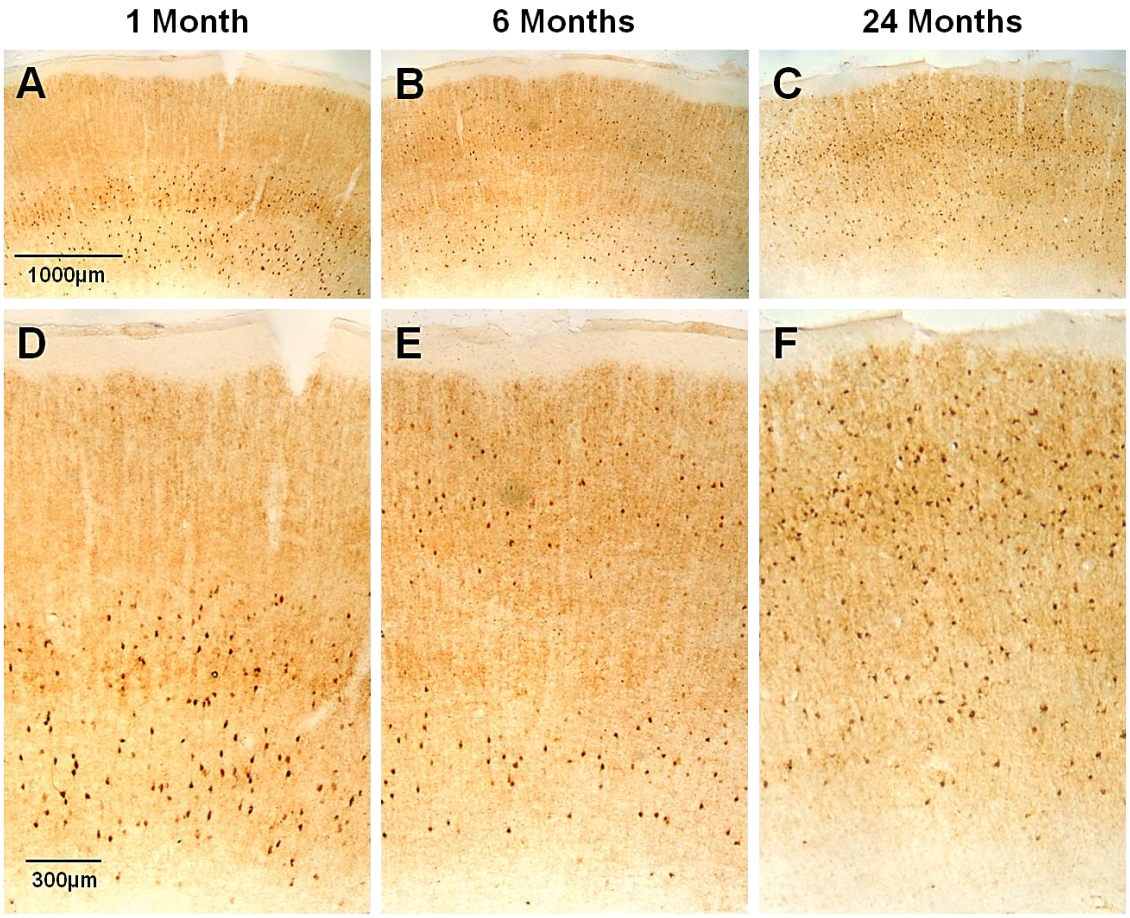
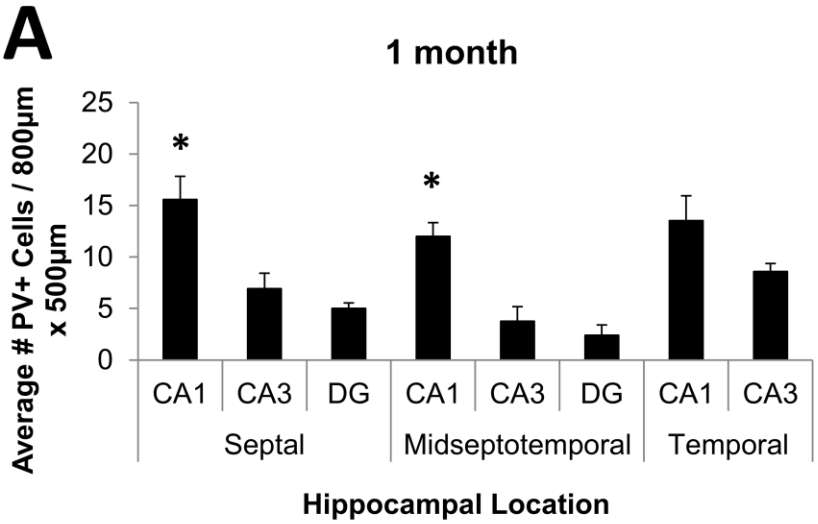
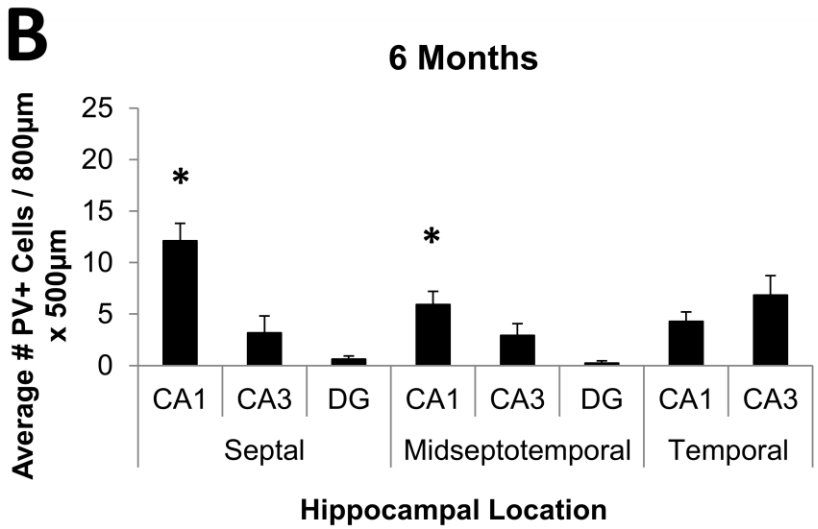


Figure 12: *PV+ expression within the somatosensory (barrel field) cortex: distribution depends on age.*

Qualitative observations within the somatosensory cortex barrel fields revealed an age-dependent distribution of PV expression across cortical layers. The top panels **(A-C)** show full photomicrographs of the barrel field cortex for 1 **(A)**, 6 **(B)**, and 24 **(C)** month old rats. It is clear that 24 month old extensively trained rats have significantly more PV+ interneurons than both 1 and 6 month old naïve rats. Bottom panels **(D-F)** show higher magnification of top panels to illustrate laminar differences in PV distribution based on age of the animal. 1 month old rats have PV expression almost exclusively in the deep cortical layers **(D)**, while 6 month **(E)** old rats (while having statistically similar overall expression to 1 month old rats) have PV expression in both deep and artificial cortical layers (but not in layers 3/4). In contrast, 24 month old rats show PV expression in all cortical layers containing cell bodies **(F)**.


A
1 month
Average # PV+ Cells / 800µm x 500µm
25
20
15
10
5
0
*
*
CA1
CA3
DG
CA1
CA3
DG
CA1
CA3
Septal
Midseptotemporal
Temporal
Hippocampal Location


B
6 Months
Average # PV+ Cells / 800µm x 500µm
25
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15
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CA1
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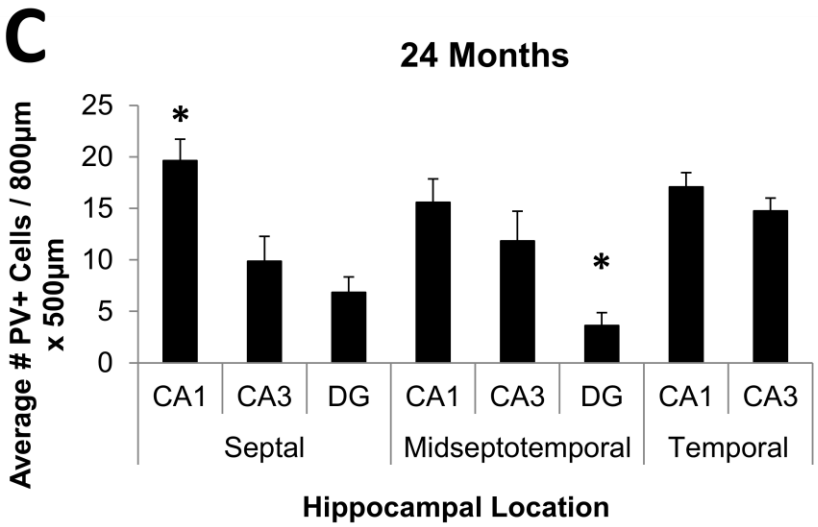
DG
CA1
CA3
Septal
Midseptotemporal
Temporal
Hippocampal Location


C
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25
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DG
CA1
CA3
Septal
Midseptotemporal
Temporal
Hippocampal Location

Figure 13: *Hippocampal CA1 has significantly more PV expression than CA3 and DG in septal and midseptotemporal regions across all age groups.*

Across all age groups (1-, 6-, and 24-months), regardless of past experience, CA1 PV expression was significantly higher in septal and midseptotemporal levels when compared to CA3 and DG **(A-C)**. Interestingly, in 24-month old extensively trained rats **(C)** there was no difference between CA1 and CA3 at the midseptotemporal level, though the DG had significantly lower PV expression than either CA1 or CA3. There was no difference between PV expression in temporal locations between CA1 and CA3 regions. *$p < .05$

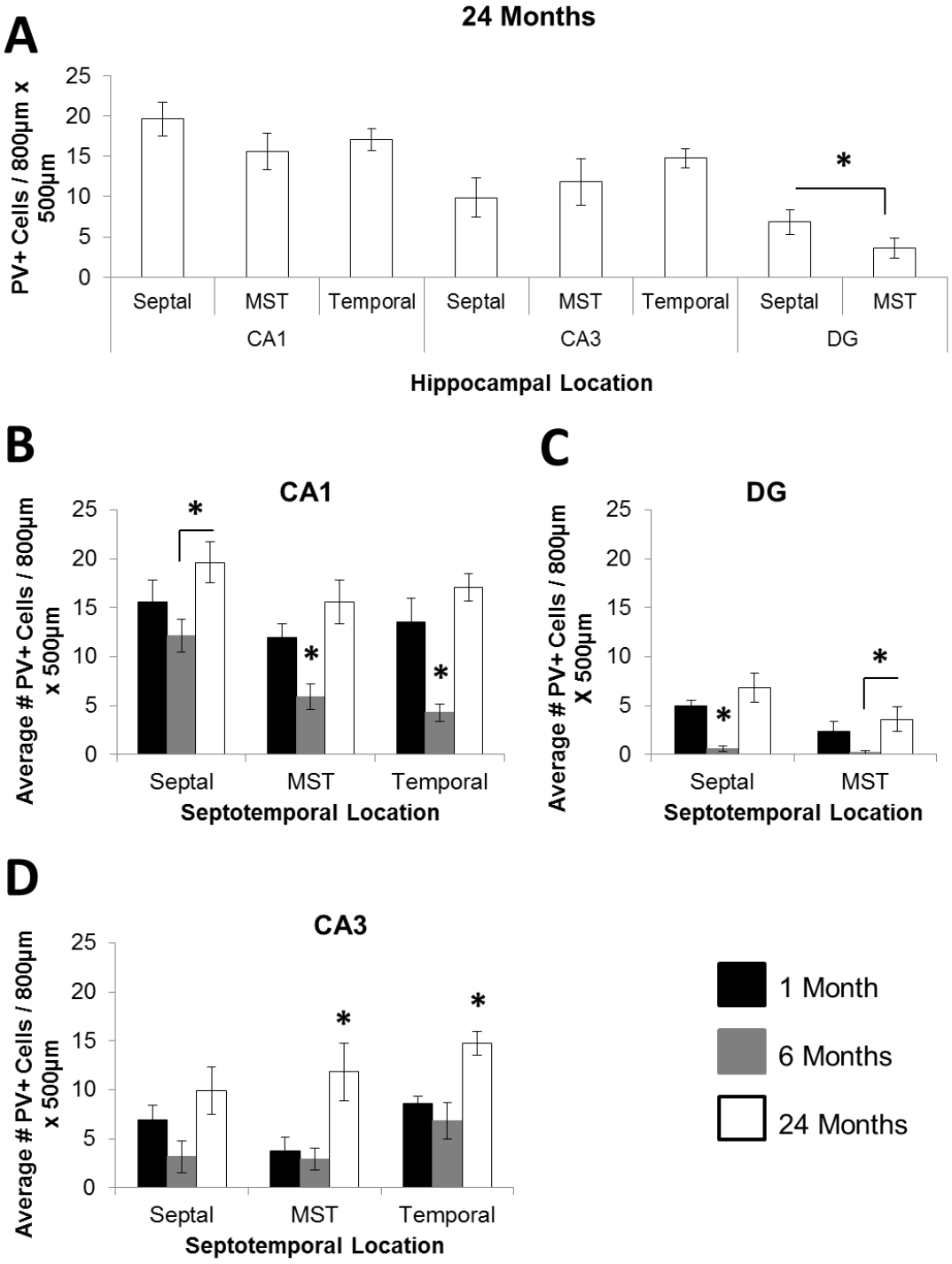

Figure 14: *24-month old extensively trained rats have elevated PV expression across all hippocampal regions.*

In CA1 and CA3, there were no septotemporal differences in PV expression in 24-month old rats; however, there was a significant decrease in PV expression between septal and midseptotemporal DG **(A)**. Within CA1 **(B)**, 24-month old

extensively trained rats had significantly higher PV expression than 6-month old naïve rats at all septotemporal levels. Furthermore, 24-month old rats had significantly higher PV expression than 1-month old rats in septal CA1, though there were no differences between the two groups in midseptotemporal or temporal CA1. Within the DG **(C)**, 1- and 24-month old rats had significantly higher PV expression than 6-month old rats in septal CA1. There were no differences between 1- and 24-month old rats at either level, and no difference between 1- and 6-month old rats in midseptotemporal DG, though a significant difference between 6- and 24-month old rats was seen in this region. 24-month old rats had significantly higher PV expression than both 1- and 6-month old rats in midseptotemporal and temporal CA3 regions **(D)**. No differences were seen between age-groups in septal CA3. *$p < .05$

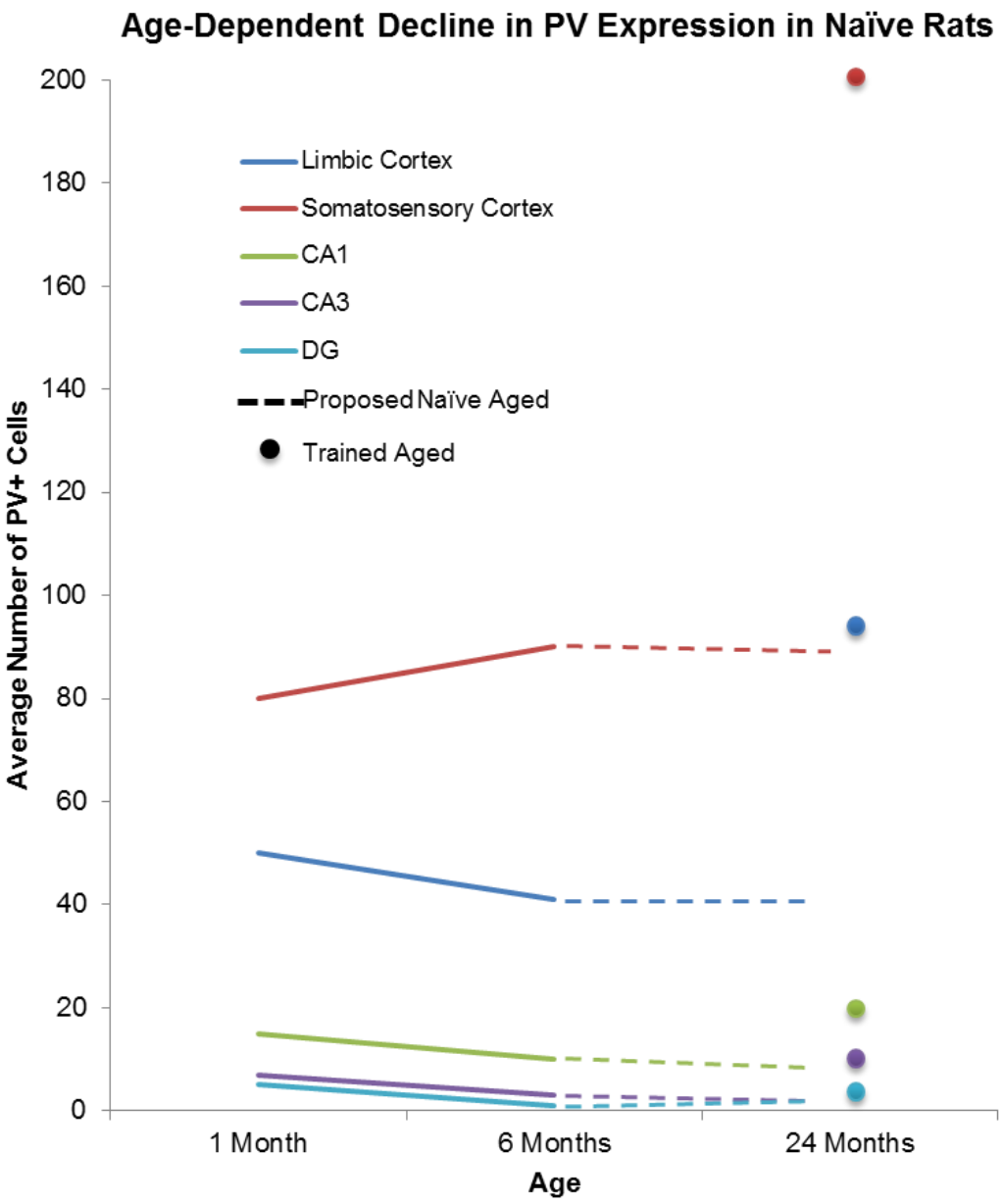

Figure 15: *Proposed age-dependent decline in PV expression as a function of brain region.*

Here, we propose a theoretical framework for PV expression as a function of age and brain region. These are approximations based on the data collected in the present study, and reflect an age-dependent decline in PV expression that continues with age, as is seen in the 1-month to 6-month decrease in hippocampal, but not cortical, areas. We propose that over the course of the lifespan, hippocampal regions (especially CA1 and DG, based on our results) would be most impacted, and CA3 would be minimally impacted, while

neocortical regions would remain relatively stable over the lifespan. Circles indicate observations seen in 24-month old behaviorally trained rats in the current study, while dotted lines indicate proposed levels of PV expression we would expect to see in naïve aged rats.

Tables

Table 1. *Average number of parvalbumin-positive interneurons across cortical and hippocampal regions in the rat brain as a function of age and septotemporal location.*

		1 month *M*±SEM	*n*	6 months *M*±SEM	*n*	24 months *M*±SEM	*n*
Cortex							
Retrosplenial [a]		50.4±8.2	8	41.5±10.7	8	95.2±21.0	8
Barrel Field [b]		73.5±18.1	8	105.4±22.0	8	250.9±31.8	8
Hippocampus[c]							
CA1	Septal	15.6±2.2	8	12.1±1.7	8	19.6±2.1	8
	Midseptotemporal	12.0±1.3	8	5.9±1.3	8	15.6±2.3	8
	Temporal	13.5±2.4	7	4.3±0.9	8	17.1±1.4	8
CA3	Septal	6.9±1.5	8	3.2±1.6	8	9.9±2.4	8
	Midseptotemporal	3.7±1.4	6	2.9±1.1	8	11.8±1.2	8
	Temporal	8.6±0.9	6	6.9±1.9	8	14.8±1.2	8
DG	Septal	5.0±0.5	8	0.6±0.3	8	6.8±1.5	8
	Midseptotemporal	2.4±1.0	6	0.3±0.2	8	3.6±1.2	8

[a] analyzed region was 1600µm x 1000µm

[b] analyzed region was 800µm²

[c] analyzed region was 800µm x 500µm